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Canine Parvovirus Capsid Assembly and Differences in Mammalian and Insect Cells

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We examined the assembly processes of the capsid proteins of canine parvovirus (CPV) in mammalian and insect cells. In CPV-infected cells empty capsids assembled within 15 min, and then continued to form over the following 1 h, while full (DNA-containing) capsids were detected only after 60 min, and those accumulated slowly over several hours. In cells expressing VP1 and VP2 or only VP2, empty capsid formation was also efficient, but was slightly slower than that in infected cells. Small amounts of trimer forms of VP2 were detected in cells expressing wild type capsid proteins, but were not seen for mutants containing changes that prevented capsid assembly. CPV capsids accumulated in the cell nucleus, but mutant VP1 and VP2 proteins that did not assemble became distributed throughout the nucleus and the cytoplasm, irrespective of whether they were expressed as VP1 and VP2, or as VP2 only. Urea or pH treatment of empty capsids released dimer, trimer, or pentamer capsid protein combinations, while treatment of full capsids consistently released trimer and, in some cases, pentamer forms. When wild type or assembly-defective VP2 genes were expressed from recombinant baculoviruses in insect cells, most of the protein was recovered as noncapsid aggregates, and only a small proportion assembled into capsids. Both the assembled capsids and the noncapsid aggregates were seen primarily in the cytoplasm of the insect cells. The VP2 expressed in insect cells that was recovered in aggregates had an isoelectric point of about pH 6.3, while that recovered from assembled capsids had a pI of about 5.2, similar to that seen for the VP2 of capsids recovered from mammalian cells.

INTRODUCTION

The assembly of capsid proteins of many icosahedral viruses follows complex pathways that involve the formation of intermediate subassemblies that subsequently form the complete capsid. Examples include the pseudo-$T = 3$ picornavirus capsids where the VP0, VP1, and VP3 assemble to form a heterotrimeric protomer (Bruneau *et al.,* 1983), five of which then associate to form a pentamer (Putnak and Phillips, 1981), 12 of which form the capsid (Ansardi *et al.,* 1996; Rombaut *et al.,* 1991). The $T = 4$ hepatitis B virus capsid assembles from dimers (Seifer *et al.,* 1993; Zhou and Standring, 1991, 1992; Zhou *et al.,* 1992), while the $T = 7$ polyomavirus capsid assembles from pentamers (Kosukegawa *et al.,* 1996; Salunke *et al.,* 1986, 1989; Sandalon and Oppenheim, 1997). However, subassemblies have not been defined for many other viruses, and it is also possible that capsid proteins of some simple spherical viruses can assemble by the varying interactions of single protein subunits (Berger *et al.,* 1994; Schwartz *et al.,* 1998, 2000; Zlotnick, 1994).

Although the structures of the $T = 1$ capsids of a number of different parvoviruses were previously determined using X-ray crystallography, relatively little is known about the pathways of capsid assembly. The capsid is assembled from two or three overlapping capsid proteins, which for most autonomous viruses are VP1 and VP2, with the smaller VP2 protein containing the structure necessary for capsid assembly (Becerra *et al.,* 1988; Cotmore and Tattersall, 1987; Rose *et al.,* 1971; Tattersall *et al.,* 1976, 1977). When only VP2 is produced from infectious clones, that protein still assembles and packages DNA, although the capsids are substantially defective for cell infection (Tullis *et al.,* 1993). Empty capsids are formed in mammalian or insect cells by expression of the VP2 of minute virus of mice (MVM) (Hernando *et al.,* 2000), canine parvovirus (CPV) (Lopez de Turiso *et al.,* 1992; Saliki *et al.,* 1992), porcine parvovirus (PPV) (Martinez *et al.,* 1992), Aleutian mink disease parvovirus (ADV) (Christensen *et al.,* 1993), mink enteritis virus (MEV) (Christensen *et al.,* 1994), duck parvovirus (DPV) (Le Gall-Recule *et al.,* 1996), and B19 (Brown *et al.,* 1991; Kajigaya *et al.,* 1991). For adeno-associated virus (AAV), VP2 is required for the formation of empty capsids when the proteins are expressed in insect cells (Ruffing *et al.,* 1992), while VP1 and VP2 can each form capsids when expressed in HeLa cells (Steinbach *et al.,* 1997).

CPV capsids are normally assembled from the VP1 and VP2, and VP1 contains the 584 residues of VP2 and 143 unique amino terminal residues (Parrish, 1991; Rhode, 1985). The CPV capsid is 26 nm in diameter, and is composed of 60 copies of a combination of VP1 and

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VP2 proteins (Tsao *et al.,* 1991; Weichert *et al.,* 1998; Xie and Chapman, 1996). In full capsids a variable proportion of the VP2 is cleaved after residue 19 by host proteases, to form VP3 (Weichert *et al.,* 1998). In CPV-infected cells empty and full capsids are formed in similar amounts.

The capsid structures of CPV, feline panleukopenia virus (FPV), and MVM show an eight-stranded antiparallel β-barrel joined by large loops, which make up most of the exposed surface of the capsid (Agbandje *et al.,* 1993, 1995; Agbandje-McKenna *et al.,* 1998; Chapman and Rossmann, 1993, 1995; Tsao *et al.,* 1991; Wu and Rossmann, 1993). Each protein monomer makes two-, three-, and fivefold related interactions with several neighbors through both the β-barrel and loop structures (Wu and Rossmann, 1993; Xie and Chapman, 1996), which may create dimers, trimers, or pentamers during the assembly process, although assembly intermediates have not yet been clearly defined.

Although VP1 and VP2 are synthesized in the cytoplasm and the capsid appears to assemble and DNA is packaged in the nucleus, there are close connections between cellular location, capsid assembly, and transport. An AAV mutant, for which capsids were not seen to accumulate in the nucleus, did not assemble (Hoque *et al.,* 1999), and mutations in MVM VP2 proteins which affected nuclear transport also affected assembly (Lombardo *et al.,* 2000), although the relationships between capsid assembly and nuclear localization are complex. A number of potential basic classical nuclear localization sequences (NLS) are present in the VP1-unique region of CPV, and one sequence between residues 4 and 13 of VP1 functions for nuclear transport when conjugated to BSA (Vihinen-Ranta *et al.,* 1997). Nuclear transport of VP2 may require formation of a trimer complex, and VP1–VP2 associations may also affect that transport in some cases (Lombardo *et al.,* 2000). The NS2 protein is required for efficient production of MVM in mouse cells as a result of an effect on capsid assembly (Cotmore *et al.,* 1997), and that associates with the 14-3-3 protein and with Crm(1), indicating a likely role in nuclear export (Bodendorf *et al.,* 1999; Brockhaus *et al.,* 1996). However, a similar role in capsid assembly was not seen for NS2 of CPV in canine or feline cells (Wang *et al.,* 1998).

Viral ssDNA is associated with the interior of the capsid and the DNA bases interact with the protein (Agbandje-McKenna *et al.,* 1998; Chapman and Rossmann, 1995). The 3′-ends of the MVM and ADV viral DNA also associate specifically when incubated with capsids of those viruses (Willwand and Hirt, 1993), and about 24 bases of the 5′-end of the DNA are exposed on the outside of the full MVM capsid, which has NS1 attached when it is first synthesized (Cotmore and Tattersall, 1989). That sequence can also be recognized on the outside of the capsid of CPV (Wang and Parrish, 1999).

Here we examined the process of capsid assembly of CPV in mammalian and insect cells. Empty capsids assembled within minutes of protein synthesis in mammalian cells, but full capsids formed only over a period of hours. A VP2 trimer was detected in lysates of cells expressing the wild type protein, but that was not seen for capsid proteins with mutations that prevented assembly. The CPV capsids were localized in the nucleus, but variant VP2, which did not assemble, was distributed throughout the nucleus and the cytoplasm. Capsids treated with high or low pH or with urea released dimer, trimer, and pentamer complexes of capsid proteins. Capsid proteins expressed alone in mammalian cells assembled into empty capsids, although the proportion of VP1 incorporated was increased threefold compared to that of capsids formed in CPV-infected cells. When VP2 was expressed from recombinant baculoviruses in insect cells only a small proportion assembled into capsids, and both the capsids and the VP2 proteins were seen primarily in the cytoplasm of those cells. Most of the baculovirus-expressed VP2 was found in aggregates that showed a higher pI than that of the assembled VP2.

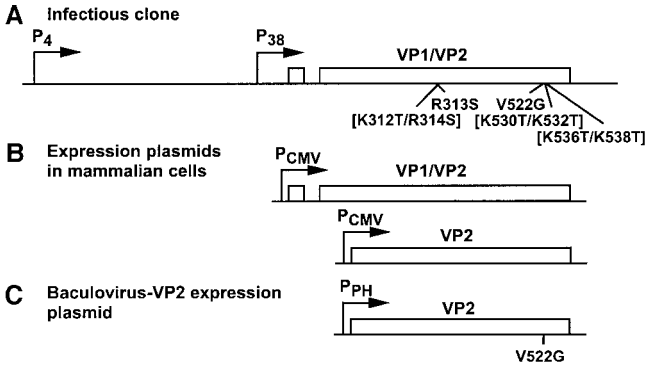

FIG. 1. Plasmids and mutants prepared for analysis of the assembly of VP1 and VP2 proteins in mammalian or insect cells. (A) Infectious plasmid clones of the CPV-d. VP1 and VP2 gene products are shown as bars, and single or pairs of mutations tested for assembly or for effects on nuclear localization are indicated as positions in the VP2 sequence. (B) VP1 and VP2 genes or VP2 gene, expressed under the control of a CMV immediate-early promoter (P_{CMV}). (C) Recombinant baculovirus clone of the VP2 gene and the VP2 V522G mutant, expressed under the control of the polyhedrin late promoter (P_{PH}).

RESULTS

Capsid assembly in mammalian cells

Capsid assembly kinetics were examined in CPV-infected A72 cells, or in A72 cell lines stably transformed with plasmids that expressed both VP1 and VP2 or only VP2, where the cells were pulsed with [^{35}S]methionine for 15 min and then the cells washed extensively before the chase period (Figs. 1A and 1B). Nonassembled VP1 and VP2 were found in the top-gradient fractions after 15 min of labeling, and then declined ~70% between 1 and 2 h, but the remainder persisted for the next 2 h of incubation (Fig. 2). In infected cells empty capsids

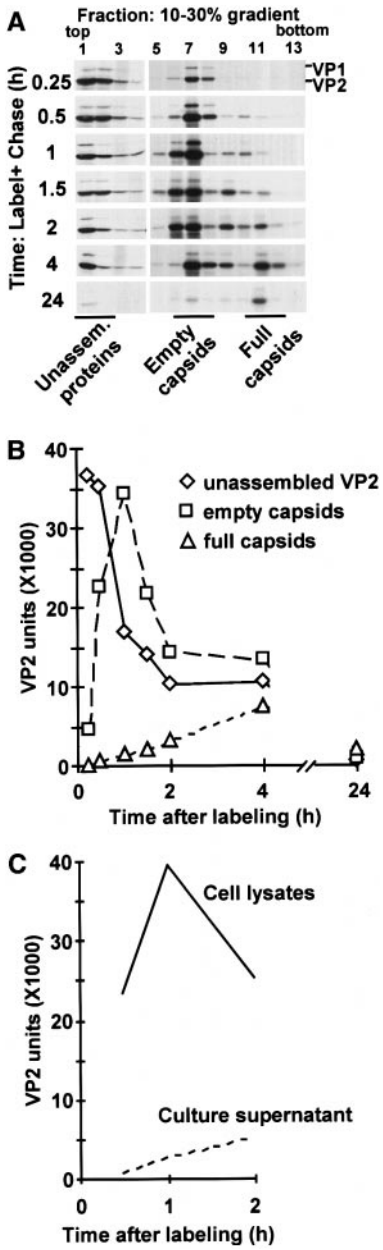

FIG. 2. Time course of expression and assembly of CPV capsid proteins in virus-infected cells. (A) Lysates of CPV-infected cells separated in 10–30% sucrose gradients. Cells were labeled with [^{35}S]methionine for 15 min, then washed and incubated in complete medium for a further 15 min to 24 h. Gradient fractions were analyzed by SDS–PAGE and fluorography. Half the amounts of the proteins were included from fractions 1 to 4 compared to the remaining fractions. (B) Quantities (phosphorimager units) of [^{35}S]methionine-labeled VP2 shown in (A), indicating amounts of VP2 in the unassembled protein, empty capsid, and full capsid fractions at various times after labeling. (C) Quantities (phosphorimager units) of [^{35}S]methionine-labeled empty and full capsids recovered between 0.5 and 2 h after labeling from the culture supernatant compared with those from the cell lysates.

formed during the initial 15 min of labeling, and maximal amounts were found by 60 min, after which the quantity declined by 40–60% (Fig. 2). Full capsids formed much more slowly and were clearly detected only after 1 h, continuing to accumulate over at least the next 3 h (Fig. 2).

In that study both the intracellular and cell-bound capsids were measured. The decrease of the amount of cell-associated empty capsids between 1 and 2 h after labeling was not accounted for by full capsid production. The possibility of capsid transport into the medium was examined by immunoprecipitation of the culture supernatant with an anti-capsid monoclonal antibody, MAb 8 (Strassheim *et al.,* 1994; Wikoff *et al.,* 1994). Small amounts of capsids were recovered from the culture supernatant 2 h after labeling. However, those accounted for less than 10% of the virus lost between 1 and 2 h of incubation (Fig. 2C), suggesting that degradation was at least partially responsible for the reduction in the amount of empty capsids and of unassembled VP1 and VP2.

When VP1 and VP2 or only VP2 were expressed from plasmids in stably transfected A72 cells, capsids were not detected after 15 min of labeling, although significant amounts were formed by 30 min (Fig. 3A). In the CPV-infected cells VP2:VP1 ratios of 9:1 were present in both the unassembled proteins and in the empty or full capsids (Figs. 2A and 3B). However, when VP1 and VP were expressed from a plasmid in A72 cells, VP2:VP1 ratios of 7:3 were found in the assembled capsids, although a 9:1 ratio was seen in the nonassembled protein fractions of those expressing cells (Fig. 3). The VP2:VP1 ratio of 7:3 was also seen for capsids purified from the same cells when analyzed by SDS–PAGE and Coomassie blue staining (results not shown).

Cellular localization of capsid proteins was related to capsid assembly

A variety of mutants were prepared from basic amino acids within possible NLS sequences (VP2 residues 312, 313, 314, 530, 532, 536, 538), or were mutants previously reported to prevent capsid assembly [Val522Gly (V522G)] (Tresnan *et al.,* 1995) (Fig. 1). Those were prepared in plasmids that expressed the infectious CPV genome, and in expression plasmids that either expressed both VP1 and VP2, or only VP2. The plasmids were tested for capsid production and for localization of the proteins and capsids after transfection of A72 cells. Capsids were detected with MAb 8, which recognizes only assembled capsids, with an antiserum against a peptide representing VP2 residues 258–270 (antipeptide 258–270), which recognizes only nonassembled capsid proteins, or with a rabbit anti-CPV antisera that recognizes both the assembled and nonassembled proteins (Strassheim *et al.,* 1994; Weichert *et al.,* 1998). Wild type VP1 and VP2 expressed in A72 cells formed capsids that were located primarily in the nucleus, and essentially no unassembled VP1 or VP2 was detected with the antipeptide 258–270 (Fig. 4A). No significant differences were seen in the

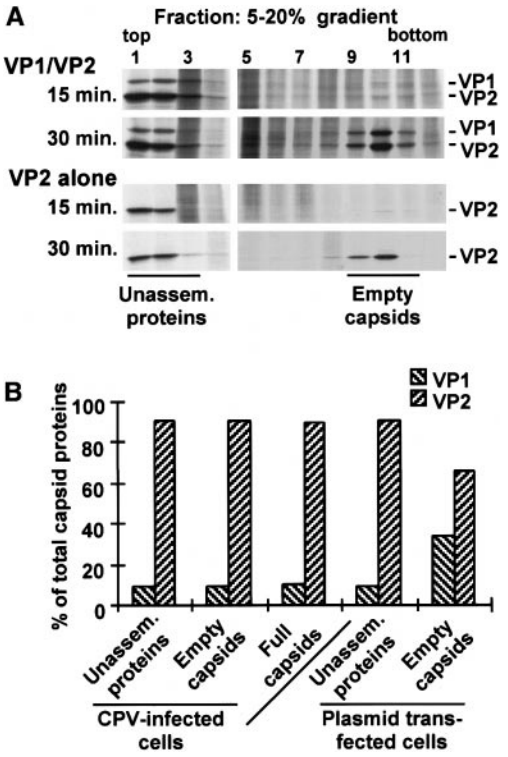

FIG. 3. (A) Protein production and capsid assembly in A72 cells transfected with plasmids expressing either VP1 and VP2 (VP1/VP2) or only VP2 (VP2 alone). Cells were labeled with [^{35}S]methionine for 15 min and the proteins were recovered, or they were chased for a further 15 min (30 min total). After centrifugation in 5–20% sucrose gradients, fractions were analyzed by SDS–PAGE and fluorography. Half the amounts of the proteins were included from fractions 1 to 4 compared to the remaining fractions. (B) Relative amounts of VP1 and VP2 (phosphorimager units) in cells either infected with CPV or transfected with a plasmid expressing VP1 and VP2. Proportions are for unassembled protein fractions (top of gradient), full capsids from infected cells, and empty capsids from infected or transfected cells.

location of the wild type proteins or capsids expressed during virus infection, from expression plasmids, or when only VP2 was expressed (Fig. 4A and results not shown). In contrast, capsid proteins with mutations which prevented assembly (V522G or K536T/K538T) were found throughout the cytoplasm and the nucleus when expressed as VP1 and VP2 or only VP2 (Figs. 4B and 4C). Mutants R313S, K312T/R314S, or K530T/K532 all formed capsids efficiently, and those were located almost entirely in the nucleus, whether they were expressed as only VP2, or as both VP1 and VP2 (Fig. 4C and results not shown).

Trimers of VP2 were detected in CPV assembly

A72 cells transfected with the infectious plasmid clones of CPV-d or with mutants V522G or K536T/K538T (Fig. 1A) were lysed and proteins separated in 10–30% sucrose gradients. Although empty and full capsids were formed from the wild type CPV plasmid clones, no capsids were detected for either of the mutants (Fig. 5A). We examined for possible assembly subunits by centrifuging for 16 h in 5–20% sucrose gradients, which separated marker proteins with molecular weights of 66, 141, 222, and 443 kDa. Analysis of lysates of CPV-d plasmid-transfected cells prepared by repeated freezing/thawing showed a separation between monomeric VP1 and VP2,

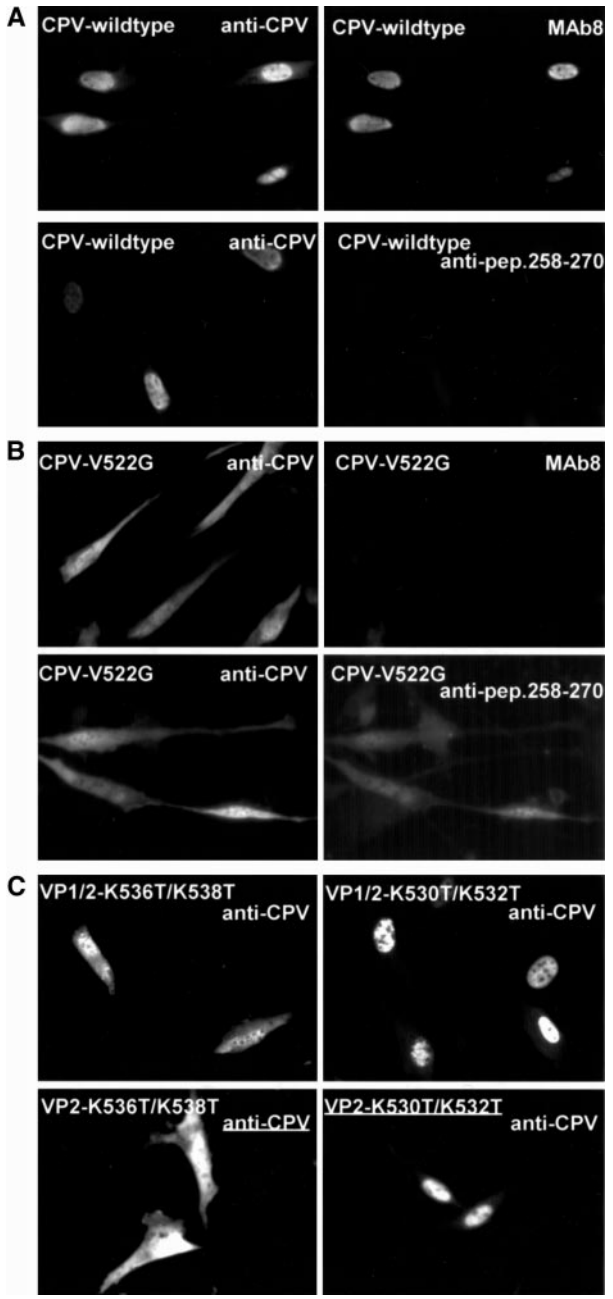

FIG. 4. Intracellular localization of CPV capsids and capsid proteins expressed from infectious plasmid clones CPV-d (A), mutant V522G (B), or mutants K530T/K532T and K536T/K538T (C). A72 cells were transfected with plasmids and then incubated for 48 h prior to fixation. Cells were stained with rabbit anti-CPV serum (anti-CPV), which recognizes both assembled capsids and unassembled VP1 and VP2, with MAb 8, or with antipeptide 258–272 antibodies.

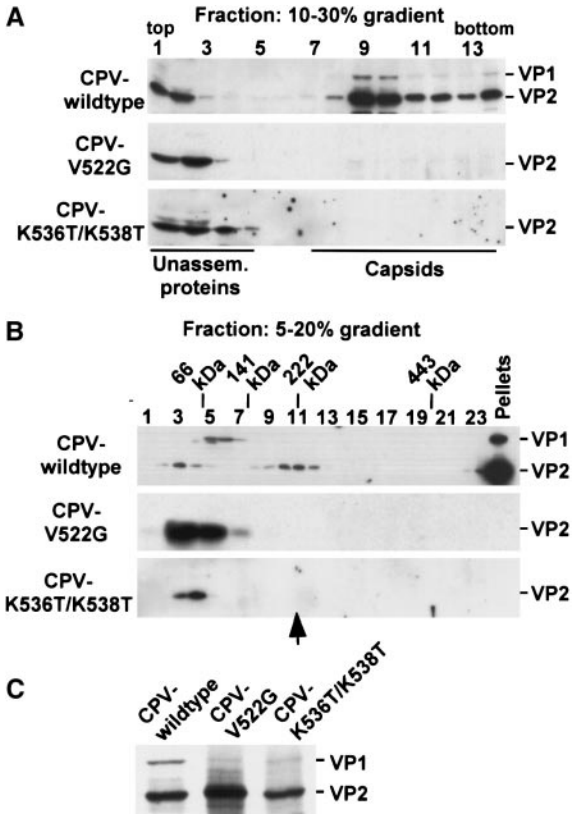

FIG. 5. Analysis of cells 48 h after transfection with plasmids expressing wild type VP1 and VP2 or the mutants V522G or K536T/K538T. (A) Cell lysates centrifuged in 10–30% sucrose gradients, showing the presence of unassembled VP1 and VP2 in the top fractions, the capsids formed by the wild type VP1/VP2, and the absence of any assembled proteins in the cases of mutants V522G and K536T/K538T. (B) Cell lysates prepared by repeated freezing/thawing were centrifuged in 5–20% sucrose gradients for 16 h at 160,000 *g*. VP1 and VP2 are indicated, as are the positions in equivalent gradients of protein standards of 66, 141, 222, and 443 kDa. The arrow indicates the predicted position of the VP2 trimer. (C) Expression and stability of VP1 and VP2 from plasmids containing the wild type CPV genome, or the mutants CPV-V522G or CPV-K536T/K538T. Plasmids were transfected into A72 cells, after which cell lysates were prepared after 15 min of labeling with [^{35}S]methionine and a 4-h chase. The total capsid proteins were precipitated with a rabbit anti-CPV antiserum.

and also showed a small amount of the VP2 sedimenting at a position of about 222 kDa in the gradient, most likely a VP2 trimer (Fig. 5B). Neither mutant V522G nor mutant K536T/K538T showed any subassemblies greater than the monomer (Fig. 5B). Little VP1 was recovered in the lysates of cells transfected with the nonassembling mutants. This appeared to be the result of degradation of the VP1 after synthesis when not incorporated into capsids because, after a 15-min pulse with [^{35}S]methionine and a 4-h chase, the VP1 of the nonassembling mutants was detected in only small amounts (Fig. 5C).

Capsid disassembly revealed subunits

Purified empty or full capsids incubated in high- or low-pH buffers, or in the presence of urea were examined by electrophoresis in 1.2% agarose gels (Figs. 6A and 6B) and by 5% SDS–PAGE (Figs. 6C and 6D). Intact capsids appeared as single bands in the agarose gel, although full capsids ran faster than empty ones (Fig. 6A). When treated with urea, full capsids started to break down in 6 M urea, and were completely disintegrated by 8 M urea (Fig. 6B). Empty capsids appeared to undergo conformational changes in the presence of 4 to 5 M urea, as double bands were seen in the agarose gel, and those disappeared when the capsids were incubated with 6 M urea (Fig. 6B). Empty capsids treated with pH $<$ 3.0 or $>$11.0, or with $>$3 M urea released VP1 and VP2 monomers, as well as complexes with the sizes of VP2 dimers, trimers, and pentamers (Figs. 6C and 6D). Although full capsids released VP1 and VP2 monomers after low-pH treatment, no multimeric forms were seen, whereas after high-pH treatment only small amounts of the trimer were seen (Fig. 6C). Urea treatment of full capsids released VP2 monomers and apparent trimers and pentamers, but did not show the dimers, which were seen after treatment of empty capsids (Fig. 6D).

Baculovirus-expressed VP2 assembled inefficiently

Although high levels of wild type VP2 were produced in Sf9 cells after recombinant baculovirus expression, little of that protein assembled into empty capsids (Fig. 7). After a 15-min pulse with [^{35}S]methionine and a 15-min chase, the wild type VP2 was spread throughout the sucrose gradient, although empty capsids formed by 30 min (Fig. 7A). In contrast, although the V522G VP2 was also found throughout the gradient, it did not form detectable capsids (Fig. 7A). Western blotting of products recovered 24 h after baculovirus infection showed VP2 throughout the gradients and, again, only wild type VP2 showed a clear peak of empty capsids (Fig. 7B). When the proteins from the gradient fractions were analyzed using MAb 8 or antipeptide 258–270 antibodies in a slot-blot assay, much of the VP2 from both preparations was unassembled, and MAb 8-reactive capsids were seen only in the wild type VP2 gradient (Fig. 7C).

The recombinant baculovirus-infected insect cells expressing wild type VP2 were stained with both rabbit anti-CPV and MAb 8 and observed by confocal microscopy. The total VP2 protein and the MAb 8-reacting capsids were both primarily found in the cytoplasm of most cells, although a small proportion of both protein forms was seen in the nucleus and a few cells showed protein production but not detectable capsids (Fig. 8).

Charge of the VP2 differed between assembled and unassembled forms

The VP2 from the nonassembled fractions from baculovirus expression and from capsids purified from mammalian cells or baculovirus-infected insect cells were

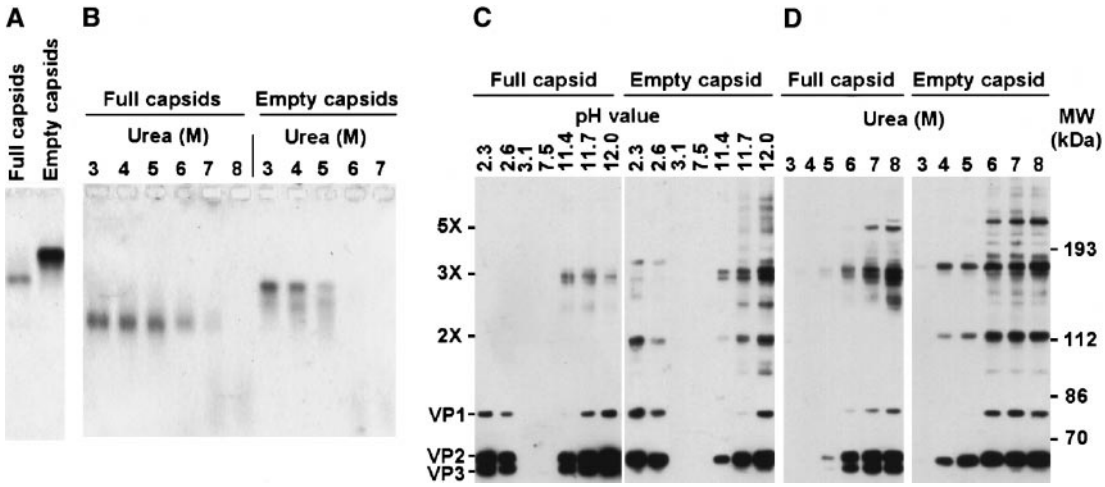

FIG. 6. Disassembly of full and empty CPV capsids after treatment with pH or urea. Purified capsids (A) and capsids treated with different concentrations of urea (B) were analyzed in 1.2% agarose gels, which were stained with Coomassie brilliant blue R250. Purified capsids were incubated in different pH buffers (C), or with different concentrations of urea (D), and then analyzed by 5% SDS–PAGE without boiling the samples. Viral proteins were detected by Western blotting with a 1:1 mixture of rabbit anti-CPV and rabbit anti-VP1/VP2 antibodies. VP1, VP2, and VP3 are indicated, and the 2×, 3×, and 5× indicate possible dimers, trimers, and pentamers of capsid proteins.

compared by isoelectric focusing electrophoresis. Several VP2 charge isoforms with pIs between pH 4.8 and 5.4 were seen in CPV-infected A72 cell extracts (Fig. 9A), or in capsids purified from CPV-infected or VP1/VP2 plasmid-transfected A72 cells (Fig. 9B). Most VP2 recovered from insect cells after baculovirus expression had a pI ~ 6.3 (Fig. 9A). However, the VP2 in capsids that were purified from the baculovirus-infected insect cells had a pI similar to that seen for empty capsids recovered from CPV-infected cells (pH ~ 5.2), while nonassembled pro-

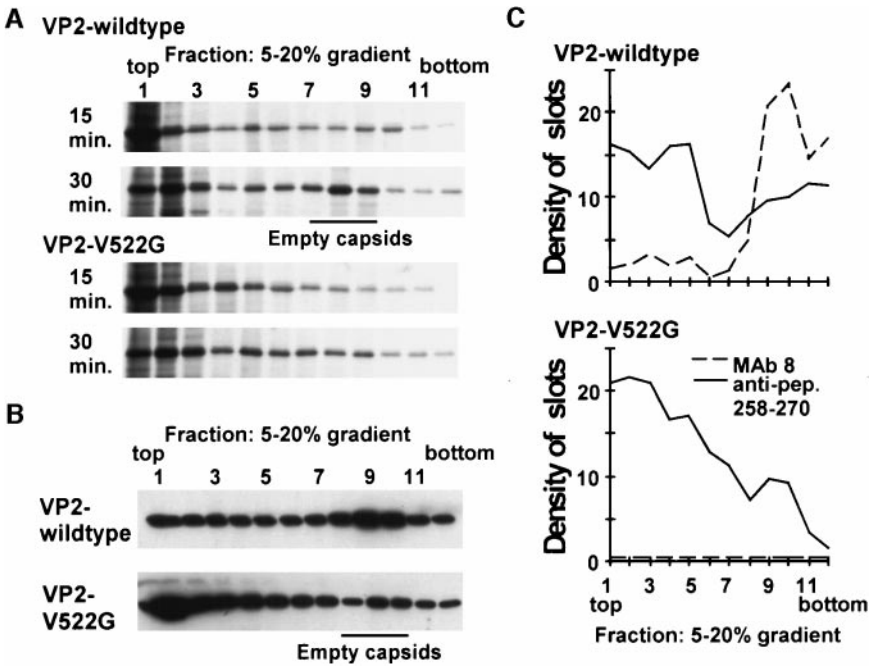

FIG. 7. VP2 aggregation or assembly after expression from recombinant baculoviruses in Sf9 cells. Clones examined expressed wild type VP2 or VP2 containing the change of V522G. (A) Pulse- and pulse-chase analysis for a total of 15 or 30 min of [^{35}S]methionine-labeled cells 24 h after recombinant baculoviruses infection. Lysates were centrifuged in 5–20% sucrose gradients, then proteins in each fraction were separated on SDS–PAGE after TCA precipitation. (B) Proteins recovered from insect cells 24 h after recombinant baculovirus infection. Proteins in gradient fractions were TCA precipitated, separated on SDS–PAGE, then Western-blotted using a rabbit anti-VP1/VP2 antiserum. (C) Analysis of proteins in gradient fractions of lysates from cells expressing wild type VP2 or VP2-V522G using structure-specific antibodies. Sucrose gradient fractions were applied to membranes in a slot-blot apparatus and proteins were detected with MAb 8 or with antipeptide 258–270 antibodies.

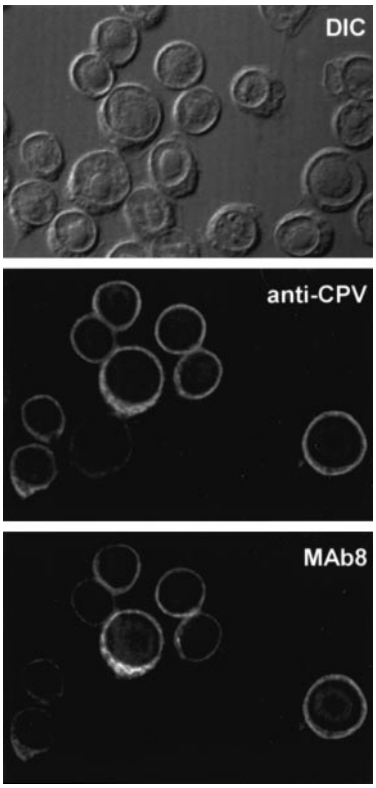

FIG. 8. Intracellular localization of VP2 proteins expressed from recombinant baculovirus and of the capsids assembled. Insect Sf9 cells were infected with recombinant baculoviruses and then incubated for 24 h prior to fixation. Total VP2 proteins were stained with rabbit anti-CPV antibodies, and the capsids detected with MAb 8. The cells were observed by confocal microscopy, and image sections were collected from the center of the cells. DIC = differential interference contrast image.

teins in fractions 1 to 3 of the sucrose gradients had a pI ~ 6.3 (Fig. 9B).

DISCUSSION

Although the structures of assembled parvovirus capsids are well understood, the details of their assembly and disassembly pathways are still not clear. Here we investigated the assembly processes of the CPV capsid in its normal host cells and in insect cells, and compared the products to those released after disassembly of normal particles. The VP1 and VP2 assembled into empty particles within 15 min of labeling, while VP1 and VP2 or VP2 expressed alone assembled into capsids at a slightly slower rate. In this case efficient capsid assembly did not require the presence of other viral components, as previously reported for studies of other parvovirus (Christensen *et al.,* 1994; Clemens *et al.,* 1992; Cohen *et al.,* 1995; Hernando *et al.,* 2000; Kajigaya *et al.,* 1991). In other viruses the NS2 protein appears to be required for efficient capsid assembly, in a host-specific manner (Cotmore *et al.,* 1997). The slightly slower rate of assembly seen after plasmid expression may be the result of the approximately 10-fold lower level of protein synthesis per cell in that case compared to that of virus-infected cells. In infected cells full capsids were detected only after 60 min of labeling, and those accumulated over at least the next 3 h. Studies of AAV and LuIII suggested that viral DNA is packaged into preformed empty capsids, and the slow formation of full capsids seen here was similar to that reported for AAV (Muller and Siegl, 1983; Myers and Carter, 1980). Whether full capsids were formed by packaging viral DNA into preformed empty capsids or from the pool of unassembled capsid proteins could not be clearly determined because, although there was a greater decrease in the empty capsid pool over the chase period, the amount of full capsid formation was relatively low (Figs. 2A and 2B). The incorporation of VP1 in expressed capsids was increased over that seen in normal empty capsids. As the proteins appeared to be expressed at the correct ratio, the overincorporation of VP1 may result from the lack of either NS1 or NS2 in those cells, causing a minor change in protein incorporation.

Intracellular localization

In cells infected with parvoviruses the virion accumulates primarily in the nucleus, and VP1 and VP2 must be transported to that site after synthesis (Lombardo *et al.,* 2000; Wistuba *et al.,* 1997). Here we saw an association between capsid assembly and nuclear localization, in that two different mutations of the VP1/VP2 gene which prevented assembly resulted in the protein being found

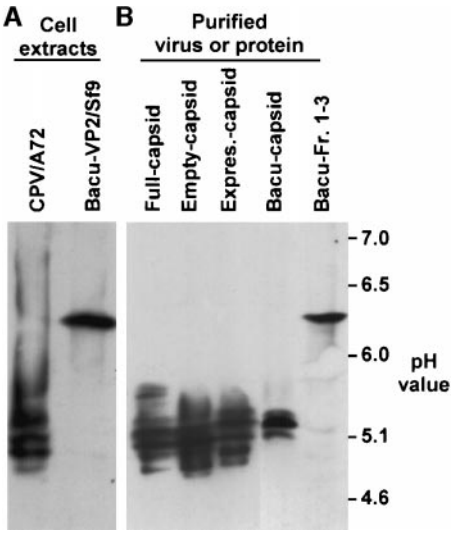

FIG. 9. Viral proteins prepared in urea and separated on isoelectric focusing gels, then detected with rabbit anti-CPV antibodies. (A) Lysates of CPV-infected A72 cells or of recombinant baculovirus-infected Sf9 cells expressing wild type VP2. (B) CPV empty and full capsids purified from infected A72 cells, empty particles from VP1/VP2-expression plasmid-transfected A72 cells, or VP2 expressed in baculovirus-infected Sf9 cells and recovered from the empty-capsid fractions or from the top three fractions of a sucrose gradient.

throughout the cell. One of those mutations (K536T/K538T) was within the motif of VP2 seen to be associated with capsid assembly and nuclear localization in MVM, but another mutation (V522G) was in a distinct region of the VP2 near the fivefold axis of symmetry. This suggests that, although the mutant VP2 entered the nucleus, it was not retained there efficiently unless it assembled into capsids. This may be because a nuclear retention sequence is displayed on the assembled capsid but not on the monomeric proteins, or because a nuclear export sequence exposed on the protein monomers is buried within the capsid after assembly (Elfgang *et al.,* 1999; Ullman *et al.,* 1997).

Studies of MVM and AAV also previously showed relationships between capsid protein assembly and nuclear localization (Hoque *et al.,* 1999; Lombardo *et al.,* 2000). For MVM, VP2 trimer formation is thought to be associated with VP2 nuclear localization (Lombardo *et al.,* 2000). Here we observed that, although two VP2 mutants did not form any detectable oligomers (Fig. 5B), they clearly entered the nucleus (Fig. 4B), indicating that the monomeric VP2 of CPV was capable of nuclear transport in CPV. The possibility that the exposed N-terminus of VP2 might be phosphorylated on three of four serines in the N-terminal sequence, leading to nuclear export of full capsids, was reported for MVM (B. Maroto, J. M. Almendral, 8th Parvovorus Workshop, Mont-Tremblant, Canada, July 2000). Only one of those serines is present in CPV, so it is not clear whether a similar transport might occur. Studies of AAV showed that, when the major capsid protein VP3 was expressed alone in HeLa cells, it became distributed between the nucleus and the cytoplasm, but did not assemble (Ruffing *et al.,* 1992). Adding the nuclear localization signal of SV40 large T antigen to the VP3 N-terminus resulted in the fusion protein assembling and becoming localized in the nucleus (Hoque *et al.,* 1999). As nuclear accumulation likely increases the local concentration of the capsid proteins, that may facilitate capsid assembly, as was previously seen for hepatitis B virus (Seifer *et al.,* 1993) and poliovirus (Rombaut and Boeye, 1991).

Assembly intermediates and subunits in disassembly

The $T = 1$ structure of the parvovirus capsid predicts possible dimer, trimer, or pentamer assembly intermediates. The trimer would likely be more stable than the other multimers, and it appears that the intertwining of loops between threefold-related proteins would have to occur before either the two- or fivefold associations could be stabilized (Xie and Chapman, 1996). When cells expressing wild type VP2 were lysed under native conditions, trimer complexes were detected (Fig. 5B), but were not seen when the cells were lysed in the presence of nonionic detergents (results not shown). The amounts of trimer present were small compared to those of the capsid, suggesting that, if the trimer is an intermediate in assembly, then it only forms transiently. When VP2 mutants with changes within fivefold interactions (V522G) or on the inside of the protein (K536T/K538T) were examined, VP2 trimers were not detected, indicating that they were not a stable intermediate that accumulated when other assembly steps were blocked.

Treatment of empty capsids with urea or with high or low pH released oligomeric forms with the sizes of dimers, trimers, and pentamers of VP2. The full capsids did not show the VP2 dimer or some of the other subassemblies seen for the empty capsid, indicating specific differences between empty and full capsids in the stability of certain VP2 multimers. These may result from the association of the viral DNA with sites between fivefold-related proteins (Chapman and Rossmann, 1995), as well as the exposure of some VP2 N-termini and the genomic 5′-end on the outside of the full capsid (Xie and Chapman, 1996).

Assembly and localization in insect cells

Although many studies of plasmid or baculovirus expression of parvovirus capsid proteins in vertebrate or insect cells were previously reported, the relative efficiency of assembly of those different expression systems has not been examined. VP2 protein expressed in insect cells assembled inefficiently, and both the VP2 and assembled capsids accumulated primarily in the cytoplasm, with only small amounts of each form being observed within the nucleus (Fig. 8). The relationship between inefficient nuclear localization and capsid assembly is difficult to define in detail. It may be that the cytoplasmic localization of the proteins resulted in the inefficient processing of the VP2, thus leading to the high pI form, which then did not assemble efficiently. In studies of MVM VP2 expression in insect cells, viral antigens were observed in the nucleus and the cytoplasm by immunoelectron microscopy, although the relative amounts and assembled forms in each compartment were not examined (Hernando *et al.,* 2000). Cytoplasmic accumulation was also seen for the polyoma virus VP2 and VP3 proteins expressed in insect cells, although those proteins were transported efficiently into the nucleus of mammalian cells (Delos *et al.,* 1993). Studies of MVM show that VP2 is found in several phosphorylated forms with different pIs, and that some of those are preferentially incorporated into full capsids (Santaren *et al.,* 1993). It is likely that the pH 6.3 isoform of CPV baculovirus-expressed VP2 results from a reduced level of phosphorylation. Although many vertebrate proteins are correctly phosphorylated when expressed in insect cells, a number of proteins were reported to be either hypophosphorylated or differently phosphorylated when compared to the same proteins expressed in mammalian

cells (de Carvalho *et al.,* 1996; Fuchs *et al.,* 1995; Jiang *et al.,* 1997).

These studies indicate that the assembly and disassembly of the parvovirus capsid are complex processes, which are regulated by the type of cell in which the protein is expressed, by whether the cell is infected or the proteins are expressed from a plasmid vector, by the requirement for nuclear transport of the VP1 and VP2, and by the interaction between ssDNA synthesis and capsid formation. In our future studies we will examine the various forms of the proteins and the mechanisms regulating capsid assembly.

MATERIALS AND METHODS

Cells and viruses

Canine A72 cells and NLFK cells were grown in a 1:1 mixture of McCoy's 5A and Leibovitz L15 media with 5% fetal bovine serum (FBS). *Spodoptera frugiperda* (Sf9) cells were grown in Grace's insect media with 3.33g/L lactalbumin hydrolysate, 3.33 g/L yeastolate, and 10% FBS. CPV was isolated from the infectious plasmid clone of CPV-d (Parrish, 1991). Viruses were grown in NLFK cells after transfection of the plasmids. Capsids were purified using previously described procedures involving precipitation with polyethylene glycol 8000, then repeated banding on either 10–40% sucrose gradients for full and empty capsids from infected cells or 5–20% gradients for empty capsids alone (Luo *et al.,* 1988; Weichert *et al.,* 1998).

Mutagenesis, virus recovery, and capsid protein expression

Mutant VP1/VP2 or VP2 genes included changes of VP2 residue 522 from Val to Gly (V522G) (Tresnan *et al.,* 1995), residue 313 from Arg to Ser (R313S), residues 312 from Lys to Thr and 314 from Arg to Ser (K312T/R314S), residues 530 and 532 from Lys to Thr (K530T/K532T), and VP2 residues 536 and 538 from Lys to Thr (K536T/K538T) (Fig. 1A). Mutants were prepared using specific oligonucleotides and uracilated ssDNA in an M13 vector (Kunkel, 1985), then the mutations were reintroduced into the VP1 or VP2 gene. Proteins were expressed either as recombinant viral genomes or under the control of the CMV promoter in the plasmids pcDNA1-neo (Invitrogen, San Diego, CA) as both VP1 and VP2 (using the CPV splice donor and acceptor sequences), or as VP2 alone derived from cDNA clones (Fig. 1B). Plasmids were transfected into A72 cells using lipofectamine (Gibco/BRL, Gaithersburg, MD), and for kinetic analysis of assembly the transformed cells were selected with 400 μg/ml of G418, and the expressing cells were cloned.

The wild type CPV VP2 gene and the VP2 gene containing the V522G mutation were used to prepare recombinant baculoviruses after cloning in the transfer vector pBlueBac4.5 (Invitrogen), downstream of the baculovirus polyhedrin promoter (Fig. 1C).

Antibodies

Monoclonal antibody 8 (MAb 8, also designated A3B10) recognizes only assembled CPV capsids (Strassheim *et al.,* 1994; Wikoff *et al.,* 1994). Rabbit antisera were against intact capsids (rabbit anti-CPV), which recognizes both capsids and unassembled capsid proteins, or against SDS-denatured capsid proteins (rabbit anti-VP1/VP2). A mouse polyclonal antiserum (antipeptide 258–270) against a peptide containing CPV VP2 residues 258–270 recognizes only nonassembled or denatured capsid proteins (Weichert *et al.,* 1998). IgGs were purified by chromatography on protein G.

Pulse-chase analysis of assembly kinetics

A72 cells infected with CPV, A72 cells expressing VP1 and VP2 or VP2 alone, or Sf9 cells infected with recombinant baculoviruses for 24 h were labeled with 0.1–0.2 mCi/ml of [^{35}S]methionine in methionine-free DMEM for 15 min, then washed three times and incubated in complete growth media for various lengths of time. The labeled cells were washed with phosphate-buffered saline (PBS) then lysed with 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.5% bovine hemoglobin, 1% Triton X-100, 2 mM PMSF, 1 mM DTT, 2 μg/ml leupeptin, 2 μg/ml pepstatin, and 2 μg/ml aprotinion at 4°C for 1 h. After clarification, the cell lysates were separated on 10–30% (CPV-infected cells) or 5–20% (plasmid expression or baculovirus-infected cells) sucrose gradients in 50 mM Tris–HCl (pH 8.0) and 150 mM NaCl, by centrifugation for 6.5 or 7 h at 100,000 *g*. Fractions were collected and proteins in the lower fractions were precipitated with 10% trichloracetic acid (TCA) at 4°C, while VP1 and VP2 in the top four fractions were recovered by immune-precipitation with a 1:1 mixture of rabbit anti-CPV and rabbit anti-VP1/VP2 antibodies using standard methods (Harlow and Lane, 1988). The TCA- or immune-precipitated proteins were boiled and analyzed on 7.5% SDS–PAGE, and the [^{35}S]-labeled VP2 protein was quantified by phosphorimager analysis.

Subunits in assembly and disassembly

To examine for subunits in assembly, A72 cells were transfected using lipofectamine with plasmids containing the infectious clones of CPV-d, or of CPV-d with mutations V522G or K536T/K538T. After incubation for 48 h the cells were lysed in DMEM by freezing/thawing three times and clarified by centrifugation, and then the supernatant was centrifuged in 5–20% sucrose gradients for 16 h at 160,000 *g*. Proteins in fractions were precipitated and analyzed by SDS–PAGE and Western blotting, and detected with rabbit anti-VP1/VP2. The migration of proteins in the gradient was calibrated using albumin (66

kDa), alcohol dehydrogenase (141 kDa), β-amylase (220 kDa), and apoferritin (443 kDa) as molecular-weight markers.

To examine for subunits in disassembly, 0.5-μg amounts of capsids were made up into 10 mM Tris–HCl buffer (pH 7.5) with between 2 and 8 M urea, or in 40 μl of 10 mM phosphate buffers (pH 2.0, 2.5, 3.0, 12.0, 12.5, 13.0), then incubated at 20°C for 30 min. Urea-treated capsids were electrophoresed in a 1.2% agarose gel in 40 mM Tris–acetate (pH 8.1) and 1 mM $MgSO_4$, then fixed and stained with Coomassie brilliant blue R250 (Duda *et al.,* 1995). To identify subunits, the pH- or urea-treated capsids were mixed with 1% SDS and 50 mM Tris–HCl (pH 6.8), and separated on 5% SDS–PAGE, after which proteins were transferred to nitrocellulose membranes and detected with a 1:1 mixture of rabbit anti-CPV and rabbit anti-VP1/VP2 antibodies.

Intracellular localization of capsids or capsid proteins

VP1 and/or VP2 genes in infectious plasmid clones or in plasmid expression vectors were transfected into A72 cells using lipofectamine. After 48 h of incubation the cells were fixed with 2.5% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100 in PBS, and stained with rabbit anti-CPV antibodies followed by Texas red-conjugated anti-rabbit IgG, or with MAb 8 or antipeptide 258–270 antibody followed by FITC-conjugated anti-mouse IgG antibodies.

The Sf9 insect cells were infected with recombinant baculovirus expressing wild type VP2 proteins. After 24 h of incubation the cells were fixed in acetone/methanol (1:1) solution, and stained as described previously. The cells were observed by confocal microscopy and images were collected from the center of the cells.

Recombinant baculovirus expression

To examine total proteins accumulating after recombinant baculovirus expression, Sf9 cells were lysed 24 h postinfection, as described above for the mammalian cells, and then the proteins were separated on 5–20% sucrose gradients. The proteins in gradient fractions were separated by SDS–PAGE, transferred to nitrocellulose membranes, and detected with rabbit anti-VP1/VP2 antibodies, followed by anti-IgG-horseradish peroxidase and chemiluminescence. Samples from each gradient fraction were also bound to nitrocellulose membranes in a slot-blot apparatus, and proteins detected with MAb 8 or antipeptide 258–270 antibodies as before. Membranes were exposed to X-ray films to give full ranges of density for each slot.

Isoelectric point comparisons

Purified viruses or virus-infected cell pellets were diluted into buffer containing 9 M urea, 4% NP-40, 2% ampholytes (pH 8 to 10), 2% 2-mercaptoethanol, 1 mM PMSF, and 5 mM NaF. Samples were separated on pH 5–8 IEF "Ready Gels" (Bio-Rad, Hercules, CA) following the manufacturer's protocols, then transferred to PVDF membranes. VP2 was then detected using a 1:1 mixture of rabbit anti-CPV and rabbit anti-VP1/VP2 antibodies and chemiluminescence.

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REFERENCES

Agbandje, M., McKenna, R., Rossmann, M. G., Strassheim, M. L., and Parrish, C. R. (1993). Structure determination of feline panleukopenia virus empty particles. *Proteins* **16,** 155–171.

Agbandje, M., Parrish, C. R., and Rossmann, M. G. (1995). The structure of parvoviruses. *Semin. Virol.* **6,** 299–309.

Agbandje-McKenna, M., Llamas-Saiz, A. L., Wang, F., Tattersall, P., and Rossmann, M. G. (1998). Functional implications of the structure of the murine parvovirus, minute virus of mice. *Structure* **6,** 1369–1381.

Ansardi, D. C., Porter, D. C., Anderson, M. J., and Morrow, C. D. (1996). Poliovirus assembly and encapsidation of genomic RNA. *Adv. Virus Res.* **46,** 1–68.

Becerra, S. P., Koczot, F., Fabisch, P., and Rose, J. A. (1988). Synthesis of adeno-associated virus structural proteins requires both alternative mRNA splicing and alternative initiations from a single transcript. *J. Virol.* **62,** 2745–2754.

Berger, B., Shor, P. W., Tucker-Kellogg, L., and King, J. (1994). Local rule-based theory of virus shell assembly. *Proc. Natl. Acad. Sci. USA* **91,** 7732–7736.

Bodendorf, U., Cziepluch, C., Jauniaux, J.-C., Rommelaere, J., and Salomé, N. (1999). Nuclear export factor CRM1 interacts with non-structural proteins NS2 from parvovirus minute virus of mice. *J. Virol.* **73,** 7769–7779.

Brockhaus, K., Plaza, S., Pintel, D. J., Rommelaere, J., and Salomé, N. (1996). Nonstructural proteins NS2 of minute virus of mice associate in vivo with 14-3-3 protein family members. *J. Virol.* **70,** 7527–7534.

Brown, C. S., Van Lent, J. W., Vlak, J. M., and Spaan, W. J. (1991). Assembly of empty capsids by using baculovirus recombinants expressing human parvovirus B19 structural proteins. *J. Virol.* **65,** 2702–2706.

Bruneau, P., Blondel, B., Crainic, R., Horodniceanu, F., and Girard, M. (1983). Poliovirus type 1 capsid polypeptides: Absence of a free form in the cytoplasm of infected HeLa cells. *Ann. Virol. Inst. Pasteur* **134,** 151–164.

Chapman, M. S., and Rossmann, M. G. (1993). Structure, sequence, and function correlations among parvoviruses. *Virology* **194,** 491–508.

Chapman, M. S., and Rossmann, M. G. (1995). Single-stranded DNA–protein interactions in canine parvovirus. *Structure* **3,** 151–162.

Christensen, J., Alexandersen, S., Bloch, B., Aasted, B., and Uttenthal, A. (1994). Production of mink enteritis parvovirus empty capsids by expression in a baculovirus vector system: A recombinant vaccine for mink enteritis parvovirus in mink. *J. Gen. Virol.* **75,** 149–155.

Christensen, J., Storgaard, T., Bloch, B., Alexandersen, S., and Aasted, B. (1993). Expression of Aleutian mink disease parvovirus proteins in a baculovirus vector system. *J. Virol.* **67,** 229–238.

Clemens, D. L., Wolfinbarger, J. B., Mori, S., Berry, B. D., Hayes, S. F., and Bloom, M. E. (1992). Expression of Aleutian mink disease parvovirus capsid proteins by a recombinant vaccinia virus: Self-assembly of capsid proteins into particles. *J. Virol.* **66,** 3077–3085.

Cohen, B. J., Field, A. M., Mori, J., Brown, K. E., Clewley, J. P., St Amand, J., and Astell, C. R. (1995). Morphology and antigenicity of recombi-

nant B19 parvovirus capsids expressed in transfected COS-7 cells. *J. Gen. Virol.* **76,** 1233–1237.

Cotmore, S. F., D'Abramo, A. M., Jr., Carbonell, L. F., Bratton, J., and Tattersall, P. (1997). The NS2 polypeptide of parvovirus MVM is required for capsid assembly in murine cells. *Virology* **231,** 267–280.

Cotmore, S. F., and Tattersall, P. (1987). The autonomously replicating parvoviruses of vertebrates. *Adv. Virus Res.* **33,** 91–174.

Cotmore, S. F., and Tattersall, P. (1989). A genome-linked copy of the NS-1 polypeptide is located on the outside of infectious parvovirus particles. *J. Virol.* **63,** 3902–3911.

de Carvalho, M. G., McCormack, A. L., Olson, E., Ghomashchi, F., Gelb, M. H., Yates 3rd, J. R., and Leslie, C. C. (1996). Identification of phosphorylation sites of human 85-kDa cytosolic phospholipase A2 expressed in insect cells and present in human monocytes. *J. Biol. Chem.* **271,** 6987–6997.

Delos, S. E., Montross, L., Moreland, R. B., and Garcea, R. L. (1993). Expression of the polyomavirus VP2 and VP3 proteins in insect cells: Coexpression with the major capsid protein VP1 alters VP2/VP3 subcellular localization. *Virology* **194,** 393–398.

Duda, R. L., Hempel, J., Michel, H., Shabanowitz, J., Hunt, D., and Hendrix, R. W. (1995). Structural transitions during bacteriophage HK97 head assembly. *J. Mol. Biol.* **247,** 618–635.

Elfgang, C., Rosorius, O., Hofer, L., Jaksche, H., Hauber, J., and Bevec, D. (1999). Evidence for specific nucleocytoplasmic transport pathways used by leucine-rich nuclear export signals. *Proc. Natl. Acad. Sci. USA* **96,** 6229–6234.

Fuchs, B., Hecker, D., and Scheidtmann, K. H. (1995). Phosphorylation studies on rat p53 using the baculovirus expression system: Manipulation of the phosphorylation state with okadaic acid and influence on DNA binding. *Eur. J. Biochem.* **228,** 625–639.

Harlow, E., and Lane, D. (1988). "Antibodies: A Laboratory Manual." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Hernando, E., Llamas-Saiz, A. L., Foces-Foces, C., McKenna, R., Portman, I., Agbandje-McKenna, M., and Almendral, J. M. (2000). Biochemical and physical characterization of parvovirus minute virus of mice virus-like particles. *Virology* **267,** 299–309.

Hoque, M., Ishizu, K.-I., Matsumoto, A., Han, S.-I., Arisaka, F., Takayama, M., Suzuki, K., Kato, K., Kanda, T., Watanabe, H., and Handa, H. (1999). Nuclear transport of the major capsid protein is essential for adeno-associated virus capsid formation. *J. Virol.* **73,** 7912–7915.

Jiang, G., Nepomuceno, L., Yang, Q., and Sladek, F. M. (1997). Serine/threonine phosphorylation of orphan receptor hepatocyte nuclear factor 4. *Arch. Biochem. Biophys.* **340,** 1–9.

Kajigaya, S., Fujii, H., Field, A., Anderson, S., Rosenfeld, S., Anderson, L. J., Shimada, T., and Young, N. S. (1991). Self-assembled B19 parvovirus capsids, produced in a baculovirus system, are antigenically and immunogenically similar to native virions. *Proc. Natl. Acad. Sci. USA* **88,** 4646–4650.

Kosukegawa, A., Arisaka, F., Takayama, M., Yajima, H., Kaidow, A., and Handa, H. (1996). Purification and characterization of virus-like particles and pentamers produced by the expression of SV40 capsid proteins in insect cells. *Biochim. Biophys. Acta* **1290,** 37–45.

Kunkel, T. A. (1985). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* **82,** 488–492.

Le Gall-Recule, G., Jestin, V., Chagnaud, P., Blanchard, P., and Jestin, A. (1996). Expression of muscovy duck parvovirus capsid proteins (VP2 and VP3) in a baculovirus expression system and demonstration of immunity induced by the recombinant proteins. *J. Gen. Virol.* **77,** 2159–2163.

Lombardo, E., Ramírez, J. C., Agbandje-McKenna, M., and Almendral, J. M. (2000). A beta-stranded motif drives capsid protein oligomers of the parvovirus minute virus of mice into the nucleus for viral assembly. *J. Virol.* **74,** 3804–3814.

López de Turiso, J. A., Cortés, E., Martínez, C., Ruiz de Ybáñez, R., Simarro, I., Vela, C., and Casal, I. (1992). Recombinant vaccine for canine parvovirus in dogs. *J. Virol.* **66,** 2748–2753.

Luo, M., Tsao, J., Rossmann, M. G., Basak, S., and Compans, R. W. (1988). Preliminary X-ray crystallographic analysis of canine parvovirus crystals. *J. Mol. Biol.* **200,** 209–211.

Martinez, C., Dalsgaard, K., Lopez de Turiso, J. A., Cortes, E., Vela, C., and Casal, J. I. (1992). Production of porcine parvovirus empty capsids with high immunogenic activity. *Vaccine* **10,** 684–690.

Muller, D.-E., and Siegl, G. (1983). Maturation of parvovirus Lu-III in a subcellular system 1: Optimal conditions for in vitro synthesis and encapsidation of viral DNA. *J. Gen. Virol.* **64,** 1045–1054.

Myers, M. W., and Carter, B. J. (1980). Assembly of adeno-associated virus. *Virology* **102,** 71–82.

Parrish, C. R. (1991). Mapping specific functions in the capsid structure of canine parvovirus and feline panleukopenia virus using infectious plasmid clones. *Virology* **183,** 195–205.

Putnak, R., and Phillips, B. A. (1981). Picornaviral structure and assembly. *Microbiol. Rev.* **45,** 287–315.

Rhode, S. L. (1985). Nucleotide sequence of the coat protein gene of canine parvovirus. *J. Virol.* **54,** 630–633.

Rombaut, B., and Boeye, A. (1991). *In vitro* assembly of poliovirus 14 S subunits: Disoxaril stabilization as a model for the antigenicity conferring activity of infected cell extracts. *Virology* **180,** 788–792.

Rombaut, B., Foriers, A., and Boeye, A. (1991). *In vitro* assembly of poliovirus 14 S subunits: Identification of the assembly promoting activity of infected cell extracts. *Virology* **180,** 781–787.

Rose, J. A., Maizel, J. V., Inman, J. K., and Shatkin, A. J. (1971). Structural proteins of adenovirus-associated viruses. *J. Virol.* **8,** 766–770.

Ruffing, M., Zentgraf, H., and Kleinschmidt, J. A. (1992). Assembly of virus-like particles by recombinant structural proteins of adeno-associated virus type 2 in insect cells. *J. Virol.* **66,** 6922–6930.

Saliki, J. T., Mizak, B., Flore, H. P., Gettig, R. R., Burand, J. P., Carmichael, L. E., Wood, H. E., and Parrish, C. R. (1992). Canine parvovirus empty capsids produced by expression in a baculovirus vector: Use in analysis of viral properties and immunization of dogs. *J. Gen. Virol.* **73,** 369–374.

Salunke, D. M., Caspar, D. L., and Garcea, R. L. (1986). Self-assembly of purified polyomavirus capsid protein VP1. *Cell* **46,** 895–904.

Salunke, D. M., Caspar, D. L., and Garcea, R. L. (1989). Polymorphism in the assembly of polyomavirus capsid protein VP1. *Biophys. J.* **56,** 887–900.

Sandalon, Z., and Oppenheim, A. (1997). Self-assembly and protein–protein interactions between the SV40 capsid proteins produced in insect cells. *Virology* **237,** 414–421.

Santaren, J. F., Ramirez, J. C., and Almendral, J. M. (1993). Protein species of the parvovirus minute virus of mice strain MVMp: Involvement of phosphorylated VP-2 subtypes in viral morphogenesis. *J. Virol.* **67,** 5126–5138.

Schwartz, R., Garcea, R. L., and Berger, B. (2000). "Local rules" theory applied to polyomavirus polymorphic capsid assemblies. *Virology* **268,** 461–470.

Schwartz, R., Shor, P. W., Prevelige, P. E., Jr., and Berger, B. (1998). Local rules simulation of the kinetics of virus capsid self-assembly. *Biophys. J.* **75,** 2626–2636.

Seifer, M., Zhou, S., and Standring, D. N. (1993). A micromolar pool of antigenically distinct precursors is required to initiate cooperative assembly of hepatitis B virus capsids in *Xenopus* oocytes. *J. Virol.* **67,** 249–257.

Steinbach, S., Wistuba, A., Bock, T., and Kleinschmidt, J. A. (1997). Assembly of adeno-associated virus type 2 capsids in vitro. *J. Gen. Virol.* **78,** 1453–1462.

Strassheim, L. S., Gruenberg, A., Veijalainen, P., Sgro, J.-Y., and Parrish, C. R. (1994). Two dominant neutralizing antigenic determinants of canine parvovirus are found on the threefold spike of the virus capsid. *Virology* **198,** 175–184.

Tattersall, P., Cawte, P. J., Shatkin, A. J., and Ward, D. C. (1976). Three structural polypeptides coded for by minute virus of mice, a parvovirus. *J. Virol.* **20,** 273–289.

Tattersall, P., Shatkin, A. J., and Ward, D. C. (1977). Sequence homology

between the structural polypeptides of minute virus of mice. *J. Mol. Biol.* **111,** 775–794.

Tresnan, D. B., Southard, L., Weichert, W., Sgro, J.-Y., and Parrish, C. R. (1995). Analysis of the cell and erythrocyte binding activities of the dimple and canyon regions of the canine parvovirus capsid. *Virology* **211,** 123–132.

Tsao, J., Chapman, M. S., Agbandje, M., Keller, W., Smith, K., Wu, H., Luo, M., Smith, T. J., Rossmann, M. G., Compans, R. W., and Parrish, C. R. (1991). The three-dimensional structure of canine parvovirus and its functional implications. *Science* **251,** 1456–1464.

Tullis, G. E., Burger, L. R., and Pintel, D. J. (1993). The minor capsid protein VP1 of the autonomous parvovirus minute virus of mice is dispensible for encapsidation of progeny single-stranded DNA but is required for infectivity. *J. Virol.* **67,** 131–141.

Ullman, K. S., Powers, M. A., and Forbes, D. J. (1997). Nuclear export receptors: From importin to exportin. *Cell* **90,** 967–970.

Vihinen-Ranta, M., Kakkola, L., Kalela, A., Vilja, P., and Vuento, M. (1997). Characterization of a nuclear localization signal of canine parvovirus capsid proteins. *Eur. J. Biochem.* **250,** 389–394.

Wang, D., and Parrish, C. R. (1999). A heterogenous nuclear ribonucleoprotein A/B-related protein binds to single-stranded DNA near the 5′-end or within the genome of feline parvovirus and can modify virus replication. *J. Virol.* **73,** 7761–7768.

Wang, D., Yuan, W., Davis, I., and Parrish, C. R. (1998). Nonstructural protein-2 and the replication of canine parvovirus. *Virology* **240,** 273–281.

Weichert, W. S., Parker, J. S., Wahid, A. T. M., Chang, S. F., Meier, E., and Parrish, C. R. (1998). Assaying for structural variation in the parvovirus capsid and its role in infection. *Virology* **250,** 106–117.

Wikoff, W. R., Wang, G., Parrish, C. R., Cheng, R. H., Strassheim, M. L., Baker, T. S., and Rossmann, M. G. (1994). The structure of a neutralized virus: Canine parvovirus complexed with neutralizing antibody fragment. *Structure* **2,** 595–607.

Willwand, K., and Hirt, B. (1993). The major capsid protein VP2 of minute virus of mice (MVM) can form particles which bind to the 3′-terminal hairpin of MVM replicative-form DNA and package single-stranded viral progeny DNA. *J. Virol.* **67,** 5660–5663.

Wistuba, A., Kern, A., Weger, S., Grimm, D., and Kleinschmidt, J. A. (1997). Subcellular compartmentalization of adeno-associated virus type 2 assembly. *J. Virol.* **71,** 1341–1352.

Wu, H., and Rossmann, M. G. (1993). The canine parvovirus empty capsid structure. *J. Mol. Biol.* **233,** 231–244.

Xie, Q., and Chapman, M. S. (1996). Canine parvovirus capsid structure, analyzed at 2.9 Å resolution. *J. Mol. Biol.* **264,** 497–520.

Zhou, S., and Standring, D. N. (1992). Hepatitis B virus capsid particles are assembled from core-protein dimer precursors. *Proc. Natl. Acad. Sci. USA* **89,** 10046–10050.

Zhou, S., Yang, S. Q., and Standring, D. N. (1992). Characterization of hepatitis B virus capsid particle assembly in Xenopus oocytes. *J. Virol.* **66,** 3086–3092.

Zhou, S. L., and Standring, D. N. (1991). Production of hepatitis B virus nucleocapsidlike core particles in Xenopus oocytes: Assembly occurs mainly in the cytoplasm and does not require the nucleus. *J. Virol.* **65,** 5457–5464.

Zlotnick, A. (1994). To build a virus capsid: An equilibrium model of the self-assembly of polyhedral protein complexes. *J. Mol. Biol.* **241,** 59–67.